Identification of VP1/2A and 2C as Virulence Genes of Hepatitis A Virus and Demonstration of Genetic Instability of 2C

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Fourteen different chimeric virus genomes were constructed from two infectious cDNA clones encoding a virulent and an attenuated isolate, respectively, of the HM175 strain of hepatitis A virus. The ability of each recombinant virus to infect tamarins and to cause acute hepatitis was determined. Comparisons of the genotype and phenotype of each virus suggested that VP1/2A and 2C genes were responsible for virulence. The 2C gene derived from the attenuated parent virus was unstable, and one or more mutations arose in this gene during the first passage in tamarins.

Hepatitis A virus (HAV) is the etiological agent of hepatitis A, an acute disease that does not progress to chronicity (17). In many developing countries, HAV infection is common and the majority of the population develops antibodies to the virus at an early age. Since naturally acquired immunity following infection apparently affords life-long protection, adults in these regions do not experience clinical hepatitis A. This is important because, although hepatitis A can and does occur in children, the infection is often subclinical or the disease is mild whereas older individuals have a more severe disease (16). HAV is enterically transmitted by ingestion of contaminated food or water. As sanitation improves in developing countries, fewer children are infected and the population of those susceptible to severe disease expands. In developed countries, much of the adult population is susceptible to HAV infection and small focal outbreaks occur periodically but large outbreaks can also occur. For instance, in Shanghai, in 1988, when about 50% of the adult population was seronegative for anti-HAV, over 300,000 young adults (average age, 28 years) developed hepatitis A following consumption of clams from local beds contaminated with HAV (24).

Four formalin-inactivated whole virus hepatitis A vaccines are currently licensed and are effective in preventing hepatitis A but require multiple doses. Unfortunately, since HAV does not replicate efficiently in cultured cells, the vaccine is expensive to manufacture. Thus, many of the developing countries, which will have an increasing need for the vaccine as sanitation improves, will not be able to afford it. For economic reasons as well as ease of administration, an attenuated vaccine would be preferable to the inactivated vaccines since smaller amounts of virus might be immunogenic and a single dose should suffice.

Indeed, an attenuated vaccine is currently used in China but it is not licensed for use elsewhere (19).

HAV is a picornavirus (17). Its genome consists of a single-stranded positive-sense RNA, 7.5 kb in length. The 5′ noncoding region (NCR) of ~735 nucleotides contains an internal ribosome entry site. The single open reading frame of the genome is translated into a polyprotein that is cleaved by a single virus-encoded proteinase to yield all but one of the individual viral proteins. The genome can be divided into three regions. The 5′ proximal region, P1, encodes four proteins, of which three (VP1, VP2, and VP3) are known to form the viral capsid. The middle region, P2, contains the 2A, 2B, and 2C genes, which encode nonstructural proteins of incompletely defined (2A, 2C) or unknown (2B) function. The 2C protein has motifs which suggest that it functions as a helicase (12), whereas the 2A protein segregates with the capsid proteins and is apparently important for particle formation (20). The 3′ proximal region, P3, encodes four proteins including 3C, the viral proteinase, and 3D, the RNA-dependent RNA polymerase.

The HM175 strain of HAV was originally isolated from the feces of a patient with acute hepatitis A (15). The wild-type virus is virulent for tamarins, a New World monkey, in which it causes significant hepatitis (21). The wild-type virus replicated extremely inefficiently in cultured cells, but adaptation to cell culture occurred during prolonged, serial passage in primary African green monkey kidney (AGMK) cells (6). The adapted virus differs from the wild type at only 22 nucleotide positions, yet it replicates significantly more efficiently in cultured cells and no longer causes severe hepatitis in tamarins (3, 18). The genomes encoding the virulent HM175 virus and its cell culture-adapted, attenuated derivative were cloned as pWt and pHAV/7 cDNAs, respectively. In vitro transcripts from each clone are infectious and, when transfected into cells in culture, produce virus that replicates to an extent indistinguishable from that of the parent virus (4, 9). Molecular recombinant
chimeras of the two viruses were used to map the mutations responsible for enhanced replication in cell culture (9). A mutation at nucleotide 3889 in the 2B gene and mutations in the 2C gene are critical for ex vivo replication in general, and additional mutations in the 5′ NCR are required for growth in certain cell lines (7, 10).

Mutations responsible for attenuation of HAV have yet to be identified. Identification of mutations that attenuate HAV would be useful for vaccine development and evaluation, but only a few such studies have been performed thus far. Various mutations in the 5′ NCR did not attenuate HM175 virus for tamarins, suggesting that this region is not important for virulence (11, 23). In contrast, replacement of the 2C gene of the human attenuated HAV/7 strain with that from a virulent strain of simian origin (AGM27) partially reversed the attenuation and resulted in a virus that did cause significant hepatitis in tamarins, suggesting that a specific nonstructural gene could alter the virulence of this virus (22). In the present study, chimeras of the virulent HM175 virus and its attenuated derivative, HAV/7, were used to identify the viral genes responsible for the virulence of this virus in experimentally infected tamarins.

MATERIALS AND METHODS

Viruses. Chimeric viral genomes were constructed from pWt and HAV/7 infectious cDNA clones by using standard molecular techniques as described previously (9). Nucleotides are numbered according to the wild-type sequence (3). The HAV/7 cDNA contained all the parental mutations with the exception of positions 7032 (silent) and 7430 (3′ NCR) which were wild type in HAV/7 but mutated in passage 32 and 35 virus (4). Infectious cDNA clones of wild-type HAV, strain HM175 (GenBank accession number M14707), and its attenuated derivative, HAV/7 (GenBank accession number M16632), were digested with restriction enzymes, or fragments were amplified from the cDNAs by PCR and appropriate gel-purified fragments were ligated to regenerate a full-length chimeric clone. In most cases, the SacI and EcoRI sites at positions 2993 and 4977, respectively, were used. The chimeric DNA clones were amplified in Escherichia coli and purified by cesium chloride density gradient centrifugation or Qiagen kits. The authenticity of all clones was confirmed by sequencing. Two mutations (at positions 4185 and 6522) were originally thought to be unique to HAV/7 (3), but due to microheterogeneity at these positions in the wild-type virus stock, both of these positions in the wild-type cDNA clone contain the nucleotide reported for the attenuated virus. In addition, clone 88Y had a unique silent mutation at 3332, which was introduced during cDNA cloning.

RNA genomes were transcribed from HaeIII-linearized plasmids with Sp6 polymerase as previously described (9). In most cases, the RNA was transfected into cultured fetal rhesus kidney cells (11-1 subclone) by the DEAE-dextran method (9). Infection was monitored by immunofluorescent microscopy, and fluorescence was amplified by reverse transcription-PCR using nested primers specific for the HM175 strain. Ten percent fecal suspensions were prepared in 10 mM Tris-HCl (pH 7.2) containing 150 mM NaCl or in phosphate-buffered saline. One hundred microliters of 10% fecal suspension was extracted with Trizol (Gibco/BRL), Pellet Paint (Novagen) carrier was added, and the RNA was precipitated with isopropanol and washed with ethanol. The entire set of samples from each animal was tested in a single run. The TaqMan system was standardized by using in vivo transcripts of HAV/7 cDNA metabically labeled by incorporation of [3H]UTP of known specific activity. As few as 10 genomes could be reproducibly detected. In the earliest experiments, animals were housed in pairs and it was therefore not possible to assign fecal specimens to a single animal. Also, the heparin used during collection of blood samples from many of the animals inhibited the PCR, so that viremia could not be determined by that method in those animals.

Sequence verification. The VPI/P2 genomic region was extracted from 100 μl of serum or from 10% fecal samples as described for the TaqMan procedure and was amplified by reverse transcription-PCR using nested primers specific for HM175. The fragment amplified by the second round of PCR was purified on agarose gels and sequenced directly (without cloning) on an automated sequencer to yield the consensus sequence.

RESULTS

Validation of parental cDNA clones. Prior to evaluation of chimeric viruses, it was important to establish that viruses recovered from pHAV/7 or the wild-type cDNA clones retained the attenuation or virulence phenotype, respectively, of the parent virus. Previously Cohen et al. had demonstrated that the virus recovered from cultures of primary or secondary AGMK cells transfected with pHAV/7 transcripts was attenuated for tamarins (2). However, in the present study, a continuous cell line (fetal rhesus kidney subclone 11-1) derived from a different species of monkey was used for recovery of virus since these cells were more permissive for replication of the wild-type as well as of the attenuated virus (9). Therefore, it was necessary to demonstrate that the virus propagated in this continuous rhesus cell line had the same attenuation phenotype as the virus propagated in the AGMK cells.

HAV/7 harvested from transfected 11-1 cells caused mild hepatitis when inoculated into a tamarin. The peak serum ICD level of 2,220 U/ml in this animal (data not shown) was comparable to those observed previously for the recombinant HAV/7 virus produced from AGMK cells transfected with pHAV/7 transcripts and attenuated for tamarins (2). However, in the present study, a continuous cell line (fetal rhesus kidney subclone 11-1) derived from a different species of monkey was used for recovery of virus since these cells were more permissive for replication of the wild-type as well as of the attenuated virus (9). Therefore, it was necessary to demonstrate that the virus propagated in this continuous rhesus cell line had the same attenuation phenotype as the virus propagated in the AGMK cells.

HAV/7 harvested from transfected 11-1 cells caused mild hepatitis when inoculated into a tamarin. The peak serum ICD level of 6,243 U/ml in one animal and 13,502 U/ml in the other (data not shown) was comparable to those observed previously for the recombinant HAV/7 virus harvested from AGMK cells transfected with pHAV/7 transcripts and propagated in 11-1 cells.

Similarly, the wild-type HM175 virus was harvested from transfected 11-1 cells and inoculated into two tamarins. The wild-type recombinant virus produced hepatitis in both inoculated animals as demonstrated by high peak serum ICD levels of 6,243 U/ml in one animal and 13,502 U/ml in the other (data not shown). The wild-type recombinant virus was clearly virulent and caused ICD elevations quite comparable to those induced by its biological wild-type parent in other studies.
(mean of four animals, 6,076 U/ml) (18). Therefore, recombinant wild-type HM175 retained its virulence phenotype when propagated in 11-1 cells.

Since each cell culture-propagated recombinant virus displayed the degree of virulence characteristic of its respective biological parent virus, it appeared to be possible to map the genes responsible for attenuation by determining the phenotypes of various chimeric viruses.

**Chimeric viruses.** The effects of 14 chimeric viruses, consisting of sequences derived from the wild-type and HAV/7 parents (Fig. 1), on tamarins were studied. The complete data set for a virulent (15Y) and an attenuated (56Y) chimeric virus is presented in Fig. 2 to illustrate the extremes of disease. The patterns shown in Fig. 2 demonstrate a number of points. First, serum levels of two liver enzymes, ICD and ALT, rose and fell in parallel. Consequently, only the ICD levels are presented thereafter because they are a more sensitive indicator of hepatitis in tamarins. Second, the peak ICD elevation occurred within a week of seroconversion. This was true for all animals that had elevated levels of serum liver enzyme in the study. Mystax 700, infected with the virulent virus, developed a somewhat more severe than usual histopathology: the only other animal in this study with a histopathology reading of 3+ was infected with the virulent 8Y clone. Fecal shedding of virus decreased around the time of seroconversion in the monkey that developed hepatitis as well as in the monkey that did not. However, in Mystax 700, as well as in some other animals in the study, viral titers of $\geq 10^4$ of feces occurred for many weeks following seroconversion and virus was occasionally detected in the feces of some animals up to 16 weeks postinoculation, the time when the experiment was usually terminated (data not shown). Peak fecal virus load was 1,000-fold lower in Mystax 773, infected with attenuated virus, than that in Mystax 700, infected with virulent virus. Similarly, the geometric mean peak virus titer was highest for the group of viruses which was the most virulent (Table 1): this group had the highest mean ICD levels, the longest period of ICD elevation, and the greatest cumulative histopathology scores.

Mystax 773, infected with the attenuated virus, did not seroconvert to anti-HAV until 8 weeks postinoculation, whereas Mystax 700, infected with the virulent virus, seroconverted after only 4 weeks of infection. A lengthy time until serocon-
version was characteristic of the most attenuated viruses. The viruses having the most attenuated phenotype took, on average, over twice as long to induce seroconversion (10 weeks compared to 4 weeks) as did those having a virulent phenotype (Table 1).

Clinical evaluation of chimeric viruses. The data for individual animals are summarized in Fig. 3. The HAV/7 and 8D recombinant viruses infected only one of two and one of three inoculated tamarins, respectively, even though each tamarin was injected intravenously with an identical aliquot of the virus

TABLE 1. Summary of data

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Geometric mean of peak/preinoculation ICD level ratio</th>
<th>Mean duration of elevated ICD levels (wk)</th>
<th>Mean histopathology suma</th>
<th>Mean time of seroconversion (wk)</th>
<th>Peak viral titer (log_{10}/g of feces)</th>
<th>Mean duration of viral titerb (&gt;10^9) (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulent</td>
<td>Both VP1/2A and 2C wt</td>
<td>10.72</td>
<td>3.33</td>
<td>11.00</td>
<td>4.00</td>
<td>8.39</td>
<td>6.33</td>
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<tr>
<td>Intermediate</td>
<td>VP1/2A wt</td>
<td>7.79</td>
<td>1.50</td>
<td>2.42</td>
<td>4.50</td>
<td>7.20</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td>2C wt</td>
<td>5.43</td>
<td>2.00</td>
<td>1.00</td>
<td>3.67</td>
<td>5.65</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Pooled resultsc</td>
<td>6.51</td>
<td>1.75</td>
<td>1.71</td>
<td>4.08</td>
<td>6.43</td>
<td>1.14</td>
</tr>
<tr>
<td>Attenuated</td>
<td>Neither VP1/2A nor 2C wt</td>
<td>2.02</td>
<td>0.57</td>
<td>0.7</td>
<td>10.57</td>
<td>5.87</td>
<td>1.71</td>
</tr>
</tbody>
</table>

aN Only scores >1 were included.

bTiters in fecal samples taken weekly.

cPooled results for VP1/2A or 2C.

d88Y not included.
stock containing approximately $10^5$ tissue culture infectious doses. Thus, these viruses appeared to be too attenuated to initiate infections consistently. All other recombinant viruses reproducibly infected the tamarins when inoculated with this dose.

The ICD and histopathology data from the infections with chimera 15Y suggested that determinants of virulence resided in the VP1/2A and 2C genes, since this chimera, which was virulent, had wild-type sequence only in these genes plus in 2B; and the one coding mutation (3889) in 2B was believed not to

FIG. 3. Summary of phenotypic characteristics of recombinant viruses in tamarins. Viruses and animals are presented in the same order in each panel as in Fig. 2. In general, two animals were inoculated with identical samples at the same time. Exceptions were 15Y and wtSE, in which one of the pair was inoculated on a different date. Tamarins were transfected intrahepatically with nucleic acid (8Y1 and 56Y) or were inoculated intravenously with virus harvested from cell cultures (all others). 36Y1 and 36Y2 represent virus harvests from two independent cDNA clones. ICD ratios ≥2 indicate hepatitis. The broad bars for fecal shedding of wild-type (wt), 8Y, and 2C viruses indicate that the origin of the feces could not be determined because animals were housed in pairs.
In order to confirm the VP1/2A and 2C mutations were responsible for the attenuation of HAV/7, the mutant virus 56Y was constructed. This virus was identical to wild-type virus except for the two mutations in VP1/2A and the four mutations in 2C, which were characteristic of HAV/7. As an additional precaution, in vivo infection was initiated by direct intrahepatic inoculation of in vitro-synthesized transcripts (8). This in vivo transfection procedure circumvented the need for transfection of cultured cells and was used to avoid accumulation of any additional mutations that might be selected during replication in cell culture. Therefore, the genomes initiating the infection in the tamarin would have a sequence identical to that of the cDNA.

As shown in Fig. 3, serum ICD levels were minimal in both tamarins infected with 56Y genomes and histopathology was absent even though both animals were infected as demonstrated by seroconversion to anti-HAV capsid protein and detection (Fig. 3) of virus in the feces of Mystax 773 (Fig. 1) and in the serum of Mystax 774 (Table 2). Therefore, these six mutations were sufficient to attenuate the wild-type virus for tamarins and to reproduce the in vivo phenotype of the HAV/7 virus.

Relative importance of VP1/2A and 2C in determining the clinical phenotype of HAV. As summarized in Table 1, VP1/2A and 2C were both critical for expression of the virulence phenotype of HAV in tamarins. However, the relative importance was not uniform across the spectrum of markers of replication and virulence. Thus, VP1/2A and 2C genes were approximately equally important in determining peak ICD values and duration of elevated ICD values and their effects were additive. In contrast, their individual effects were minimal in determining histopathology cumulative scores, but the two genes together were highly associated with more-severe and prolonged histopathologic changes. VP1/2A but not 2C was associated with high fecal titers of HAV, but the highest titers were detected when both genes were of wild-type origin and acted synergistically. Interestingly, neither gene alone appeared to have an effect on the duration of high-level (greater than 10⁶ genomes per ml) viral replication but, when both were of wild-type origin and acted synergistically. Finally, each gene independently was associated with the phenotype of maximally shortened incubation period. The two genes therefore acted independently for determining extent and duration of ICD elevations and shortened incubation period, whereas they acted synergistically to determine histopathology and viral fecal load and duration.

Sequence analysis of selected viruses recovered from infected tamarins. The region spanning VP1/2A through 2C was amplified by reverse transcription-PCR from virus excreted in the feces of representative infected animals. Sequence analysis of the PCR product representing the highly virulent virus 15Y demonstrated that the 15Y virus excreted by two different animals retained the exact sequence of the cDNA (Table 2).
Two animals that were infected with 36Y2, in which the 2C gene was of the wild type, shed virus in which the 2C gene retained the wild-type sequence although the 2B gene acquired a silent (3992) and a coding (3841) mutation (Table 2). Since the viruses recovered from the two animals contained those identical mutations, they undoubtedly were acquired during the cell passage step. The wild-type virus had acquired a single new mutation which changed an amino acid in 2C (Table 2), but since two animals were housed together, it was not possible to determine if the mutation was also present in the virus infecting the second animal. In contrast, animals infected with a highly attenuated virus excreted virus that, in all seven cases examined, had accumulated one or more nonsynonymous mutations in 2C compared to the original cDNA clone (Table 2). In three different viruses (HAV/7, 8D, and 1H, recovered from Mystax 683, 691, and 753, respectively), the identical mutation was selected: this mutation at position 4563 in 2C caused the encoded amino acid to revert from that found in the attenuated virus to that present in the original wild type.

The multiple reversions of nucleotide 4563 suggested that this mutation might be very important for infection of tamarins. Therefore, a chimeric virus (88Y) that was identical to the highly attenuated 56Y virus (Fig. 1) except for having wild-type nucleotides at positions 4043 (silent mutation) and 4563 (He reverted to Val) was tested. Following intrahepatic transfection of two tamarins with genomes from this chimera, the ICD levels in each animal became elevated for 1 week with peak ICD ratio values of 4.01 and 6.89, respectively, suggesting an intermediate level of virulence. Seroconversion occurred at weeks 8 and 9, respectively. The sequence recovered from each animal was identical to that in the cDNA clone (Table 2); most strikingly, new mutations were not acquired in 2C.

Two mutant viruses that had been selected in the tamarins were tested directly for reversion to virulence. The virus excreted by Mystax 757 following infection with 1H virus, and that excreted by Mystax 773 following infection with 1H virus were inoculated into two tamarins each. These viruses were chosen because each parent virus had been highly attenuated and each mutant had acquired a unique mutation in 2C. Each of the four animals developed anti-HAV earlier than had animals infected with the parent virus, and in all four animals serum liver enzymes were elevated (Table 3). Therefore, each of these new mutations increased the virulence of the virus.

**DISCUSSION**

Previous studies with chimeric viruses containing genomic regions from two very different HAV strains, one of human origin and one of simian origin, had suggested that the 2C gene of the simian strain was a determinant of virulence (22). This much more comprehensive comparison of the phenotypes of 14 different chimeric viruses containing many combinations of genes from wild-type HM175 virus or an attenuated derivative of the same human strain demonstrated that the ability of HAV to cause acute hepatitis in tamarins depended on the genotypes of both the VP1/2A and the 2C genes. A virus was virulent if these genes were from the wild-type virus, and conversely, a virus was attenuated if these genes contained the six mutations found in the HAV/7 virus. Other regions of the HM175 genome did not have a discernible effect on virulence in this study.

Tamarins have been used for many studies of HAV because they provide a good small-animal model in which to study the virus and the disease it causes (21). The differences observed between two animals infected with identical inocula most likely reflect a combination of biological variation and limitations in the number of serum and biopsy samples that could be obtained. Because of the small size and fragile nature of tamarins, serum samples and liver biopsies were collected only once a week and peak liver enzyme values and histopathology might therefore have been missed, resulting in an underestimation of the extent of hepatitis. Also, since the needle biopsies monitored only a tiny fraction of the liver, any focal lesions would be difficult to detect. In spite of these caveats, the patterns that emerged, shown in Fig. 3, were remarkably consistent and clearly demonstrated that both VP1/2A and 2C genes were important in determining the severity of acute hepatitis.

The data summarized in Table 1 suggest that both VP1/2A and 2C wild-type genes are required for maximum virulence. Viruses with this genotype caused the most-severe hepatitis, as indicated by both the highest ICD levels and the greatest histopathology scores (Fig. 3; Table 1). However, if either the VP1/2A or the 2C gene was wild type and the other gene was attenuated, serum ICD levels were still significantly elevated although they were not as high as when both genes were wild type. Thus, each gene appeared independently to increase serum liver enzyme levels. In contrast, induction of severe histopathology appeared to require that both genes be wild type since this was the only group with consistently high biopsy scores. However, it should be cautioned that the biopsy samples were very small and in some cases were difficult to read or contained scar tissue rather than liver cells, so the biopsy data were less comprehensive than were the data for other parameters measured. At this time there is not a good explanation for those cases such as 18Y in which histopathology was minor or absent even though ICD levels were quite high. Overall, the data suggested that either the VP1/2A or the 2C gene alone was less likely to cause severe histopathology than it was to induce serum liver enzyme elevations, although it must be noted that the two animals with the highest ICD levels in the VP1/2A wild-type group and the animal with the highest ICD level in the 2C wild-type group also had the highest histopathology scores in their groups.

The pattern of fecal shedding in general was consistent with previous assumptions that more-severe hepatitis reflected higher levels of viral replication. The geometric mean virus titer was the highest and remained high for the longest period
for the group of animals with the most-severe hepatitis. However, it was possible for viral fecal titers to reach as high as $10^6$ in the absence of extensive serum liver enzyme elevations, as in the case of the animal infected with HAV/7.

There are not enough data about the functions of the VP1/2A and 2C proteins to permit a conclusion as to how they cause hepatitis. The 2C protein contains helicase and nucleotide binding motifs (12) and accumulates in membraneous replication complexes (13), suggesting that it participates in RNA replication (17). The HAV/7 mutations in 2C promote efficient growth of the HM175 strain in cell culture (7); however, in the tamarins, if VP1/2A was attenuated, the mean peak fecal titer was the same whether the 2C gene was wild type ($5.65 \log_{10}$ genomes/g of feces) or attenuated ($5.87 \log_{10}$ genomes/g of feces), suggesting that 2C by itself did not control the level of viral replication in vivo (Table 1). Ex vivo, 2C mutations are most effective when combined with the 3889 mutation in 2B, which is essential for efficient replication in cultured cells, but the data in the present study suggest that the 2B gene did not affect either virulence or fecal shedding. In contrast, the VP1/2A mutations do not play a detectable role in growth in cell culture whereas they had a central role in attenuation. The VP1/2A junction is apparently not cleaved by the viral proteinase, but rather, 2A is probably degraded by a cellular proteinase so that VP1 is eventually released (14). The 2A portion of the VP1/2A precursor is required for virion formation but is not incorporated into the virion (20). The mutation in VP1 is just upstream of the VP1 carboxy terminus, so it is possible that it affects protein processing and/or virion morphogenesis. Regardless of the mechanisms involved, it appears that the 2C gene plays a critical role for replication ex vivo and for virulence in vivo and that, in both cases, the 2C protein functions in concert with a second protein. This second protein is 2B ex vivo but VP1/2A in vivo, suggesting that different processes are affected.

One of the most intriguing results was that the time from inoculation to seroconversion was consistently shorter for all animals having received virus with either the VP1/2A or 2C wild-type gene than for animals that received virus containing neither wild-type gene (Fig. 3; Table 1). In most cases, there did not appear to be a correlation between peak titer or duration of virus shedding and time to seroconversions. For instance, the viruses in which 2C was wild type and VP1/2A was attenuated were shed to a similar extent as were the viruses in which both 2C and VP1/2A were attenuated, yet the latter group of viruses required a prolonged incubation period to induce seroconversion. The sequence analysis of viruses recovered from the animals offered a possible explanation.

The data from the chimera studies suggest that any attenuated vaccine candidate would most likely need mutations in both VP1/2A and 2C. However, analysis of viruses recovered from animals suggests that viruses with the four attenuating mutations in 2C are not genetically stable. In all seven cases that were examined, a mutation in 2C that either was unique or represented a reversion of nucleotide 4563 to the wild type was selected. The reversion of nucleotide 4563 was of particular interest. Of the three coding mutations in 2C, those at 4087 and 4222 were previously shown to be important for accelerated growth in cell culture but that at 4563 appeared not to be required (7). The reversion of nucleotide 4563 in three of six infected animals suggests that the wild-type nucleotide at this position provided a strong selective advantage in vivo. The amino acid sequence of the 88Y virus differed from that of 56Y at only one amino acid position, that specified by nucleotide 4563. However, in contrast to the highly attenuated 56Y virus, the 88Y virus induced intermediate ICD levels comparable to those in animals infected with viruses in which the entire 2C gene was wild type: therefore, the 4563 mutation impacts virulence and, of the three coding mutations in 2C of HAV/7, it may be the only one which has a discernible effect in vivo.

The apparent genetic stability of the 2C region in vivo if the sequence was wild type compared to the instability if it was attenuated provides a possible explanation for the prolonged time to seroconversion for those animals infected with attenuated virus. We originally assumed that a low level of virus replication resulted in a slow humoral immune response. An alternative explanation suggested by the sequence data is that the level of replication in the fully attenuated virus was initially too low to induce humoral immunity but enough to permit selection of a mutation that resulted in increased replication, which thereby induced antibody. Since in most of these cases enzyme elevations were not detected, either the mutations did not restore virulence (unlikely, given the reversion of nucleotide 4563 and the results obtained with 88Y, which indicated that this mutation was able to restore intermediate virulence) or the amount of replication prior to the mutation was sufficient to prime the immune system so that when viral replication increased, the antibody response was rapid enough to prevent hepatitis. The failure of the HAV/7 and 8D viruses to induce seroconversion in one of two and in two of three animals, respectively, could thus be explained by failure to mutate an appropriate site in a timely fashion. The results also suggest that the level of virus replication observed reflected mostly that of the mutated virus and did not necessarily reflect that of the original virus. It is worth noting that the sequence analysis of the virus recovered from Mystax 774 confirmed that it was the original 56Y construct but that it had acquired a new mutation. Since seroconversion did not occur until week 22, the virus persisted in this animal for over 5 months before it triggered a detectable humoral immune response.

Overall, these studies suggest that an attenuated vaccine might not be safe although it might protect the vaccinee. The two tamarins inoculated with the mutated virus excreted by Mystax 773 or Mystax 757, respectively, seroconverted much sooner than did those which had received the parent virus, and more importantly, in all four animals the mutant virus caused hepatitis whereas the parent virus had not. Also, the 88Y genome, basically a 56Y genome in which the 4563 mutation was molecularly reverted to the wild type prior to infection, was of intermediate virulence, whereas the 56Y virus was more attenuated. By extrapolation, the viruses shed by tamarins 683, 691, and 753, in which the 4563 site reverted in vivo, would be predicted to cause some disease upon infecting a second host since their VP1/2A and 2C proteins would be identical to those of 88Y. Therefore, the data suggest that a single passage through an individual might be sufficient for reversion to virulence and could produce virus that posed a health hazard to contacts of the vaccinee. If mass immunizations in which all susceptible individuals within a population were vaccinated simultaneously were performed, as has been done for oral
poliovirus vaccine, the problem of secondary infections would be minimized. However, immunization of a few individuals or only a portion of a cohort might provide a vehicle for spreading virulent HAV.

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